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Regenerative fluorescence "turn-on" probe for biothiols through Cu(II)/Cu(I) redox conversion

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ABSTRACT

We present a regenerative molecular sensor for biothiols where a non-fluorescent copper(II) coumarin complex ($Cu^{II}L$) probe turns to a strongly fluorescent Cu(I) complex by thiol-containing amino acids (Cys, Hcy, GSH). During the recognition process, Cu(II) ion of $Cu^{II}L$ undergoes chemical reduction followed by substitution of the coordinating ligands with the biothiols. Interestingly, the strongly fluorescent Cu(I) complex, which quantifies the amount of the target biothiol, was oxidized back to its original $Cu^{II}L$ by chemical (or electrochemical) oxidation, and could be repeatedly reused as a thiol probe for several turn-overs. $Cu^{II}L$ was successfully applied in fluorescent imaging of the cellular GSH.

1. Introduction

Biothiols such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) are known to play an important role in a myriad of vital cellular processes, including redox homeostasis [1,2] and cellular growth [3,4]. Alterations in the cellular levels of biothiols are linked to various chronic diseases such as arthritis, cancer, and HIV/AIDS [5,6]. Considerable attention has been paid to the detection of biothiols using analytical techniques such as mass spectrometry [7], gas chromatography [8], and high-performance liquid chromatography [9] coupled with spectrophotometric or electrochemical methods [10,11]. Recently, fluorescent probes have been used for detecting biothiols due to their simplicity, high selectivity, and good sensitivity [12,13]. Most of fluorescent methods, however, are based on irreversible chemical reactions between the probe and the thiol providing only a single-use assay: Michael addition [14–17], cyclization with aldehyde [18,19], or a cleavage of sulfur-containing bond by the thiol [20].

Herein, we report a unique molecular probe for biothiol detection, wherein the probe undergoes a series of chemical reactions during target recognition (Scheme 1). A synthetic probe, copper(II) coumarin complex (**Cu^{II}L**), obtained by combining an equimolar mixture of $CuCl_2$ and the ligand (L), selectively binds to biothiols, generating strong fluorescence which is caused by the chemical reduction of reduction of Cu(II) into Cu(I) during the binding event. Interestingly, Cu(I) ion can be oxidized to Cu(II) ion by the addition of *N*-ethylmaleimide (NEM), which completely quenches the fluorescence again, and this fluorescence "off/on" cycle is observed repeatedly for several turn-overs without a significant decrease in the fluorescence intensity.

2. Experimental

2.1. Materials and instruments

Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. Chromatography was carried out on silica gel 60 (230–400 mesh ASTM; Merck). Thin layer chromatography (TLC) was carried out using Merck 60 F_{254} alumina plates with a thickness of 0.25 mm. ^1H NMR and ^{13}C NMR spectra were recorded using Bruker 300 or Varian 200. Mass spectra were obtained using a JMS-HX 110A/110A Tandem Mass Spectrometer (JEOL). UV absorption spectra were obtained on Agilent 8453 Double Beam UV/VIS Spectrometer. Flu-

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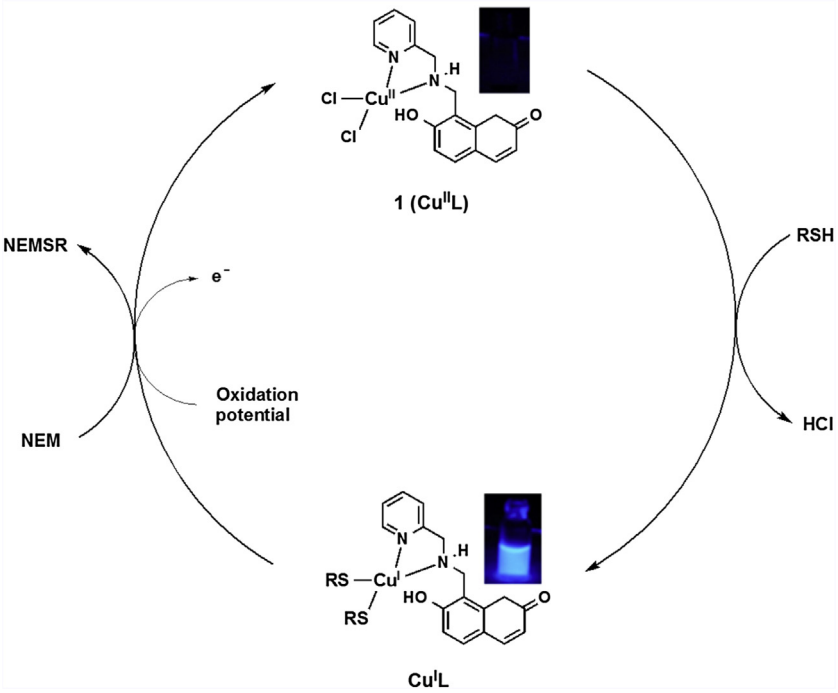

Scheme 1. A proposed mechanism for regenerative fluorescence sensing of biothiols.

orescence emission spectra were obtained using JASCO FP-6500 Spectrofluorometer.

2.2. Synthetic procedure

2.2.1. Synthesis of ligand

To a solution of coumarin aldehyde [21] (200 mg, 1.05 mmol) in EtOAc (10 mL) was added 2-picolylamine (120 mg, 1.1 mmol). Resulting clear yellow solution was further stirred at room temp. for 1 h to afford yellow precipitates, which were filtered off, washed with cold EtOAc, and dried in air to obtain the imine intermediate. The yellow solid was further reduced by sodium triacethoxyborohydride without further purification. To a solution of the intermediate (200 mg, 0.71 mmol) in CH_2Cl_2 (10 mL) was added $NaHB(OAc)_3$, (190 mg, 0.89 mmol). Resulting clear yellow solution was further stirred at room temp. for 12 h, which was purified by column chromatography using dichloromethane/MeOH (10:1 v/v, $R_f = 0.40$) as an eluent. Evaporation and drying in vacuum afforded the desired product in 85% yield over 2 steps. ^{1}H NMR (DMSO-d_6, 300 MHz): δ/ppm 9.08 (s, 1H), 7.90 (d, $J = 9.02$ Hz, 1H), 7.77 (s, 1H), 7.42 (d, $^3J = 6.85$ Hz, 2H), 7.28 (s, 1H), 6.71 (d, $J = 7.98$ Hz, 1H), 6.15 (d, $J = 8.99$ Hz, 1H), 4.07 (s, 2H), 3.89 (s, 2H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ/ppm 163.26, 160.84, 158.54, 153.44, 149.38, 145.53, 137.19, 128.61, 122.81, 122.72, 113.87, 110.96, 110.74, 110.40, 53.35, 42.90. HRMS (FAB$^+$, *m*-NBA): *m*/*z* obs'd 283.1088 ([M + H]$^+$, 283.1083 cal'd for $C_{16}H_{14}N_2O_3$)

2.2.2. Synthesis of $Cu^{II}L$ (probe 1)

To a solution of free ligand (38 mg, 0.10 mmol) in 1 mL CH_3CN was added $CuCl_2$ (0.10 mmol) in 1 mL MeOH. Resulting blue solution was further stirred for 1 h at room temp. to afford a light blue precipitates, which were filtered off, washed with CH_3CN (2 × 1 mL) and dried in vacuum. Yield 70% (Scheme 2). MS (ESI, positive): *m*/*z* obs'd 344.02 (cal'd 344.02 for [M−Cl]$^+$).

Single crystals of **Cu^{II}L** suitable for the X-ray crystallography were obtained by slow evaporation of diethylether into a MeOH/water solution of **Cu^{II}L** at room temperature. Diffraction data were collected on a Bruker SMART X-ray diffractometer at room temperature using graphite-monochromated Mo-Kα radiation (λ = 0.71073 Å). The structures were solved by direct methods (SHELXS-97), and refined against all F^2 data (SHELX-97). All non-hydrogen atoms were refined with anisotropic thermal parameters and the hydrogen atoms were treated as idealized contributions. Crystallographic data in CIF format for **Cu^{II}L** is contained in CCDC No. 1013353.

2.3. Preparation for fluorescence study

A stock solution (10 mM) of **Cu^{II}L** in DMSO was prepared and used by dilution in aqueous DMSO solution for in vitro and in vivo fluorescence experiments. In a typical experiment, test solutions were prepared by placing 2 μL of the probe stock solution into a test tube, adding an appropriate amount of each amino acid, and diluting the solution to 2 mL with buffer (0.10 M HEPES, pH 7.4). Normally, excitation was at 325 nm. Both the excitation and emission slit widths were 3 nm × 3 nm. Fluorescence spectra were monitored after addition of amino acids.

2.4. Fluorescence cell imaging of HeLa cells

For the detection of biothiols in live cells, HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units/mL penicillin, 100 g/mL streptomycin, and 10% heat-inactivated fetal bovine serum. The cells were seeded on a Ø 35 mm glass-bottomed dish at a density of 1×10^5 cells in a culture medium overnight for live-cell imaging by confocal laser scanning microscopy (CLSM). The HeLa cells were treated with 10 μM of **Cu^{II}L** in 2 mL of serum free medium for 0.5 h and washed with 3 times with pre-warmed 1 × PBS before imaging by CLSM.

2.5. Electrochemistry

Electrochemical studies were performed using a CH Instruments 650B Electrochemical Analyzer (CH Instruments, Inc., Texas, USA). All electrochemical studies were referenced with respect to an Ag/AgCl (sat'd with KCl) reference electrode. A glassy carbon disk